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(71) Applicant: BEHRINGWERKE Aktiengesellschaft  
35001 Marburg (DE)

(72) Inventors:

- Knapp, Bernhard, Dr.  
D-35083 Wetter (DE)
- Hungerer, Klaus-Dieter, Dr.  
D-35041 Marburg (DE)
- Bröker, Michael, Dr.  
D-35041 Marburg (DE)
- von Specht, Bernd Ulrich Prof.  
79353 Bahlingen/Kaiserstuhl (DE)
- Domdey, Horst Prof.  
82061 Neuried (DE)

(54) **Immunogenic hybrid protein OprF-OprI derived from Pseudomonas aeruginosa membrane proteins**

(57) The present invention relates to a hybrid protein comprising the *Pseudomonas aeruginosa* outer membrane protein I (OprI) which is fused with its amino terminal end to the carboxy-terminal end of a carboxy-terminal portion of the *Pseudomonas aeruginosa* outer membrane protein F (OprF), as well as to monoclonal or polyclonal antibodies against this hybrid protein. Both, the hybrid protein and the antibodies directed to the hybrid protein confer protection against an infection by *Pseudomonas aeruginosa* to laboratory animals or man.

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## Description

5 The present invention relates to a hybrid protein comprising the *Pseudomonas aeruginosa* outer membrane protein I (OprI or OMPI) which is fused with its amino terminal end to the carboxy-terminal end of a carboxy-terminal portion of the *Pseudomonas aeruginosa* outer membrane protein F (OprF or OMPF), as well as to monoclonal or polyclonal antibodies against this hybrid protein. Both, the hybrid protein and the antibodies directed to the hybrid protein confer protection against an infection by *Pseudomonas aeruginosa* to laboratory animals or man.

10 *Pseudomonas aeruginosa* is an opportunistic gram-negative pathogen. It represents a major cause of hospital-acquired infections, especially in burnt and other immuno-compromised patients, including transplant or cancer patients. Therefore, it is regarded as a "problem microbe" in human medicine.

15 Many efforts have been made so far in order to develop a vaccine against *Pseudomonas aeruginosa*. For example, in the EP-0 297 291 the complete amino acid sequence of the outer membrane protein F, as well as the nucleotide sequence coding for OprF is disclosed. In the EP-0 357 024 the complete amino acid sequence of the outer membrane protein I and, additionally, the nucleotide sequence coding for OprI is shown. Furthermore, with both proteins it was shown that they may be useful for conferring immuno protection against *Pseudomonas aeruginosa* to an animal or human proband. However, improvement of procedures of vaccination against a lethal *Pseudomonas aeruginosa* infection is still an object.

20 Surprisingly, it was found by the inventors that a hybrid protein, wherein OprI is linked with its N-terminal end to a C-terminal portion of OprF is significantly more immunogenic than fusion proteins only comprising OprI or OprF or mixtures of the latter: fusion proteins.

25 Thus, the present invention relates to a hybrid protein comprising the *Pseudomonas aeruginosa* outer membrane protein I which is fused with its amino-terminal end to the carboxy-terminal end of a carboxy-terminal portion of the *Pseudomonas aeruginosa* outer membrane protein F, said carboxy-terminal portion comprising the sequence from aa 190 to aa 350. In a preferred embodiment said carboxy terminal portion is the sequence from aa 190 to aa 342.

30 The present invention further relates to a hybrid protein comprising the *Pseudomonas aeruginosa* outer membrane protein I which is fused with its amino terminal end to the carboxy-terminal end of a carboxy-terminal portion of the *Pseudomonas aeruginosa* outer membrane protein OprF, wherein said carboxy-terminal portion comprises one or more of the surface-exposed B-cell epitopes SEE 1, SEE 2, SEE 3 and SEE 4. These B-cell epitopes are located at the following amino acid (aa) positions of the OprF: SEE 1 = aa 212-240, SEE 2 = aa 243-256, SEE 3 = aa 285-298 and SEE 4 = aa 332-350 (see example 1 and Hughes et al. (1992), Infect. Immun. 60, pp. 3497-3503).

35 Another embodiment of the present invention is a vaccine comprising at least one of the above-mentioned hybrid proteins.

40 Moreover, the present invention relates to monoclonal or polyclonal antibodies directed to one or more of the above hybrid proteins. These antibodies may also be used in a vaccine in order to confer passive protection against an infection by *Pseudomonas aeruginosa* to a subject.

Further aspects of the present invention are nucleic acids which are coding for the above-mentioned hybrid proteins.

45 Additionally, the present invention relates to a process for the preparation of the above-mentioned hybrid proteins, which comprises bringing about the expression of a nucleic acid as mentioned above, which is coding for a hybrid protein according to the invention, in pro- or eukaryotic cells.

40 The invention is further explained in detail in the examples which follow and in the claims.

45 In the following the sources of the microorganisms and the DNAs as well as methods that were used in the following examples, and which are for example regarded useful for carrying out the invention are indicated.

50 **Microorganisms:** *P. aeruginosa* International Antigenic Typing Scheme serogroup I (ATCC 33348) was obtained from A. Bauernfeind, Max. von Pettenkofer-Institut, University of Munich. Bacteria were grown and adjusted to the required concentration as previously described (Finke, M. et al. (1990), Infect. Immun., 58, pp. 2241-2244). For the expression of recombinant proteins *E. coli* K-12 W3110 lac<sup>Q</sup>L8 was used. For expression of OPRs in yeast we used *Saccharomyces cerevisiae* strain HT393 (leu2, ura3 prb1, prc1, pre1, cps1).

55 **Source of DNAs:** Three recombinant plasmids were used as the source of DNAs: pFSaul, a pUC19 derived plasmid that contains a 1,0 kb Sau 3AI-fragment of the *P. aeruginosa* outer membrane protein F gene encoding the C-terminal part of the protein from amino acid positions 57 to 350 (Duchêne, M. et al. (1988), J. Bacteriol. 170, pp. 155-162); pITaq1, a pUC19 derived plasmid that contains a 626 bp TaqI-fragment spanning the complete OprI gene (Duchêne, M. et al. (1989), J. Bacteriol. 171, pp. 4130-4137), and the expression vector pGEX-2a originating from the vector pGEX-2T modified by the introduction of the polylinker from vector pTRC. The vector pGEX-2a contains the tac promoter followed by the coding sequence for 26 kDa *Schistosoma japonicum* glutathione-S-transferase, a cleavage site for thrombin and the pTRC specific polylinker region.

55 **Characterization of antisera induced against synthetic peptides:** Synthetic peptides representing amino acid regions 190-213 (D1), 212-240 (D2, SEE 1), 239-250 (D3), 284-316 (D4), and 332-350 (D5, SEE 4) from OprF were synthesized as described in (Roussilhon, C. E. et al. (1990) Immunol. Lett. 25, pp. 149-154). Rabbits were immunized subcutaneously at eight different locations near lymph nodes with 200 µg KLH conjugated peptide in complete Freund's

adjuvant, and reimmunized two weeks later with 400 µg of the conjugate in incomplete Freund's adjuvant. The animals received two booster injections intravenously of 150 µg and 100 µg of conjugate six and nine weeks after the first immunization. Antibody titers against peptides were measured by ELISA on plates coated with 5 ng per ml of peptide solution in 50 mM sodium phosphate buffer, pH 7.5 (PBS) overnight at room temperature. Plates were washed three times with 5 0.05 M citric acid and 0.05 M Tris, pH 7.4, and then dried over silica gel for 3 days. Rabbit sera were diluted 1:160 and saturated with *E. coli* proteins. Western blot analysis with recombinant GST fusion-proteins and immunofluorescence determinations against intact *P. aeruginosa* serogroup 11 (ATCC 33358), were carried out by a method reported in the literature (Johnson, D. A. et al. (1984) Gene Anal. Techn. 1, p. 3-8) Schnorr, J. B. et al. (1991), Vaccine 9, pp. 675-681).

5 **Expression of OprF and Oprl as glutathione-S-transferase, fusion proteins:** The oligonucleotides p1 (5'-AAA GAG CTC GCT CCG GCT CCG GAA CCG GTT GCC GAC-3') with a SacI restriction site at the 5' end, corresponding to bases 568 to 594 of the OprF gene, and p2, (5'-AAA AAG CTT ACT TGG CTT CGG CTT CTA CTT CGG-3') with a HindIII restriction site at the 5' end, complementary to bases 1028 to 1053 of the OprF gene, and 10 ng of the plasmid pFSau1 were employed for a polymerase chain reaction, using the Perkin Elmer Cetus Gen-Amp Kit, which yielded a 500 bp fragment. The amplified fragment was digested with SacI and HindIII and introduced into the vector pGEX-2a to obtain plasmid pGEX-OprF, which encodes the C-terminal part of the porin OprF from amino acids 190 to 350. The oligonucleotides p3 (5'- CGT ACC ATG GTG AGC AGC CAC TCC AAA GAA ACC GAA GCT-3'), with an Ncol restriction site at the 5' end corresponding to bases 61 to 87 of the coding region of the Oprl gene, and p4 (5'-AAA AAG CTT CTA TTA CTT GCG GCT GGC TTT TTC C-3'), with a HindIII restriction site at the 5' end complementary to bases 231 to 255 of the coding region of the Oprl gene, and 10 ng of the plasmid DNA pITaq1 were used in a polymerase chain 10 reaction to amplify a 215 bp fragment, which was then treated with the restriction enzymes Ncol and HindIII to introduce it into the corresponding sites of the expression vector pGEX-2a, in order to obtain plasmid pGEX-Oprl, which encodes amino acids 21 to 83 of Oprl.

15 20 25

**Construction of the GST-Oprl-OprF and GST-OprF-Oprl hybrid genes:** The oligonucleotides p1 (see above) and p5 (5'-TTC AAC GCG ACG GTT GAT AGC GCG-3') (which is complementary to bases 1003 to 1026 of the OprF gene) and 10 ng of the plasmid pFSau1 were used to amplify a 470 bp OprF fragment A, second polymerase chain reaction was carried out with 10 ng of plasmid pITaq1 and the oligonucleotides p4 (see above) and p6

30 (5'-GAA GGC CGC GCT ATC AAC CGT CGC GTT GAA AGC AGC CAC TCC AAA GAA ACC GAA GCT-3'),

35 in which nucleotides 1 through 30 correspond to bases 997 to 1026 of the OprF gene and nucleotides 31 through 57 correspond to bases 61 through 87 of the Oprl coding region. This yielded a 240 bp fragment. 150 ng of both obtained DNA fragments and oligonucleotides p1 and p4 were used for a third polymerase chain reaction as described by Horton (Horton, R. M. et al. (1989), Gene 77, pp. 61-68). The obtained 660 bp fragment was digested with the restriction endonucleases SacI and HindIII, and introduced into the vector pGEX-2a to obtain plasmid pGEX-OprF-Oprl, which encodes amino acids 190 to 342 of OprF and amino acids 21 to 83 of Oprl. The oligonucleotides p3 and p7 (5'-AAA GAG CTC 40 CTT GCG GCT GGC TTT TT CAG CAT GCG-3') with a SacI restriction site at the 5' end, complementary to bases 223 to 249 of the coding region form the Oprl gene, and 10 ng of plasmid pITaq1 were used to amplify a 210 bp fragment, which was introduced into the vector pGEX2a with the help of the restriction enzymes Ncol and SAcl. The obtained plasmid was digested with the enzymes SacI and HindIII to introduce a 490 bp fragment obtained by digestion of the plasmid pGEX-OprF, using the corresponding enzymes. Plasmid, pGEX-Oprl-OprF encodes amino acids 21 to 83 from Oprl and amino acids 190 to 350 from OprF, which are separated by a two amino acid linker introduced at the SacI cloning site.

45 **Expression and purification of the recombinant proteins in *E. coli*:** The four plasmids pGEX-OprF, pGEX-Oprl, pGEX-OprF-Oprl and pGEX-Oprl-OprF were transformed into the *E. coli* K-12 strain W3110 lac I<sup>Q</sup>L8. For large scale antigen production, 5-liter bacterial cultures containing the plasmids were left to grow to OD<sub>660</sub>=1 and the expression 50 of the *P. aeruginosa* specific recombinant antigens induced by isopropylthiogalactoside. After disruption of the cells the four different glutathione-S-transferase fusion proteins were found to be soluble in aqueous solutions. Therefore, the four fusion proteins could be purified from crude bacterial lysates under non-denaturing conditions by affinity chromatography on immobilized glutathione to a purity of about 80 %.

55 **Active immunization and protection experiments:** 4 groups (A-D) of 68 female BALB/c Mice (10-12 weeks old) each received 100 µg of antigen: GST (A), GST-OprF + GST-Oprl (B), GST-OprF-Oprl (C) or GST-Oprl-OprF (D), suspended in 100 µl of "ABM 2 complete" as adjuvant, (Sebak, Aidenbach) on day 0. Booster injections were given with an equal amount of antigen suspended in 100 µl Al(OH)<sub>3</sub> on days 14, 28 and 42. On day 49 animals were bled from the tail vein for serum collection to determine antibody titers in the pooled sera of 7-10 mice from each group. Four days later, all the animals received immunosuppressive treatment. For immunosuppression mice received three injections of

150 µg cyclophosphamide (Serva, Heidelberg, Germany) per g of body weight in 0.25 ml of phosphate-buffered saline (PBS) on days 53, 55, 57. On day 58, each antigen group was divided into 4 subgroups, I, II, III, IV, containing 16-17 animals per subgroup. The mice of groups A-D received intraperitoneally either  $5 \times 10^1$  (subgroup I),  $5 \times 10^2$  (subgroup II),  $5 \times 10^3$  (subgroup III) or  $5 \times 10^4$  (subgroup IV) CFU of *P. aeruginosa* serogroup 1. 15 additional nonimmunized mice underwent only immunosuppression without bacterial challenge. This control group was used to confirm the state of leukopenia and to exclude nonspecific infections. All surviving animals were monitored for 10 days after infection.

**Expression and purification of recombinant OprF-OprI in yeast:** For expression of the *P. aeruginosa* outer membrane proteins in *S. cerevisiae* the yeast/*E. coli* shuttle vector pYepsec1 (Baldari, C. et al. (1987) EMBO J. 6, pp. 229-234) was used. This plasmid expresses polypeptides fused to the signal sequence of the *Kluyveromyces lactis* killer toxin. The Ncol/HindIII DNA fragment from pGEX-OprF-OprI, which codes for the OprF-OprI hybrid protein, was isolated, and cloned into pYepsec1, cut with BamHI and HindIII (yielding pYepsec1-F-I). The Ncol and BamHI sites were turned into blunt ends with Klenow enzyme before ligation, whereas the HindIII site was not treated. The soluble OprF-OprI hybrid protein expressed in yeast was purified by affinity chromatography, using a monoclonal antibody directed against epitope D1. The MAb was coupled to BrCN activated sepharose 4B (Pharmacia, Freiburg, Germany), in accordance with the instructions of the manufacturer. Yeast extracts in PBS were loaded onto the column, unspecific bound material was eluted with 0.1 M glycine pH 9.0 buffer containing 0.5 M NaCl. Elutions of OprI-OprF hybrid protein was carried out in 0.1 M glycine buffer, pH 11.0. The column was regenerated by washing with 0.1 M glycine, pH 2.5, followed by washing with PBS.

**Production of specific immunoglobulins and passive immunization:** Rabbits were immunized three times with 100 µg of purified recombinant OprF-OprI isolated from *S. cerevisiae* cell extracts (or with cell extracts from *S. cerevisiae* alone as controls) emulsified in incomplete Freund adjuvant on days 0, 14 and 28. On day 38, blood samples were obtained and allowed to clot overnight at 4 °C. The serum was removed, centrifuged and stored at -20 °C. In groups of 30 female SCID mice (18-20 g, Bomholtgard, Denmark), every animal in the group received either 0.5 ml of rabbit anti OprF-OprI serum or 0.5 ml of rabbit anti yeast serum. As an additional control, the animals in one group received 0.5 ml of normal saline. Those in one additional group were injected with 0.5 ml of rabbit serum against heat inactivated cells of serogroup 1 of *P. aeruginosa*. After 3 hours, the animals of groups 1-6 were subdivided into 5 subgroups (a-e), receiving 0.5 ml of *P. aeruginosa* serogroup 1 suspension ( $10^1$ ,  $10^2$ ,  $10^3$ ,  $10^4$ ,  $10^5$  CFU/ml suspended in mucin respectively. The surviving animals were observed for 1 week. 5 g mucin (Sigma, Taufkirchen, Germany) were suspended in 100 ml of distilled water, treated for 10 min. with an Ultra Turrax blender, passed through a sieve and autoclaved for 15 min at 120 °C. Shortly before use, the solution was adjusted to pH 7.2-7.4 with sterile 1N NaOH.

## Examples

### Example 1: Epitope mapping of OprF.

In order to identify amino acid sequence sections of OprF representing B-cell epitopes as a rational basis for the choice of an Opr-based *P. aeruginosa* vaccine, we prepared monoclonal antibodies against a recombinant protein representing amino acids 58 to 350 of OprF. Binding of the MAbs was analyzed with a series of recombinant subfragments of OprF expressed in *E. coli*. The MAbs discriminated between 5 different regions: aa 190-213 (D1), aa 212-240 (D2, SEE 1), aa 239-250 (D3), aa 284-316 (D4) and aa 332-350 (D5, SEE 4). The C-terminal part of OprF between aa 190 and aa 350 seemed therefore to cover most of the B cell epitopes of OprF. To further analyze the epitopes, synthetic peptides related to the above defined amino acid sections were prepared and conjugated to KLH. Polyclonal antisera against these peptides were raised in rabbits. Table 1 shows that peptides D1-D5 were recognized by the corresponding polyclonal antisera. The peptides D1, D2, D4 and D5 reacted with monoclonal antibodies, and peptides D2, D3, D4 and D5 were also recognized by polyclonal antibodies raised against recombinant OprF, thus confirming that these 5 epitopes are B-cell derived. Antisera raised against D3, D4 and D5 recognized OprF in Western blot analysis, but viable *P. aeruginosa* cells showed positive fluorescence only after incubations with the antisera raised against D2 and D5. These two epitopes therefore seem to be surface-exposed. Additional MAbs were identified which did not react with any of the synthetic peptides, but recognized GST-OprF and further recombinant subfragments, leading to two additional epitopes, D6 and D7, which correspond to amino acid residues 240-316 and 190-250 respectively. Therefore, the region from amino acid 190 to amino acid 350 of OprF was considered to include important antigenic regions, and we decided to ascertain whether recombinant proteins carrying these epitopes are able to confer protection in animal models.

### Example 2: Epitope mapping of OprI.

With the MAbs 2A1, 6A4 and 5B4 raised against native OprI, two different epitopes have been characterized (Finke, M. et al. (1991), Infect. Immun. 59, pp. 1251-1254). MAb 2A1, which had shown protective ability against *P. aeruginosa* infection, recognized the N-terminal located epitope. Subsequent studies showed that 2A1 only binds if the entire amino acid sequence from amino acid 21 to amino acid 83 is expressed. For the construction of recombinant OprI antigens as

means of a subunit vaccine, the complete amino acid region 21-83 was therefore considered to be the most adequate antigen.

**Example 3: Expression of Oprs in *E. coli*.**

5 The efficacy of a single outer membrane protein of *P. aeruginosa* in a vaccine against *P. aeruginosa* infection might be improved by coexpression of the fused epitopes of two different Oprs. Four different glutathion-S-transferase-fusion proteins were expressed in *E. coli* in large amounts: GST-OprF<sub>(aa 190-350)</sub>, GST-OprI<sub>(aa 21-83)</sub>, GST-OprF<sub>(aa 190-342)</sub>, OprI<sub>(aa 21-83)</sub> and GST-OprI<sub>(aa 21-83)</sub>-OprF<sub>(aa 190-350)</sub> (Fig. 1). The recombinant proteins could be about 80 % purified by 10 affinity chromatography on immobilized glutathion. Western blot analysis of the four recombinant products with the OprI specific Mabs 6A4 and 2A1 and different OprF specific Mabs directed against the epitopes D1, D2, D4, D5, D5, D6 and D7 showed that the MAb specific epitopes were expressed by the recombinant fusion proteins.

**Example 4: Active immunization with *E. coli* derived fusion proteins.**

15 Mice were immunized four times at two week intervals with 100 µg of recombinant GST linked fusion protein, or GST only, suspended in adjuvant "ABM complete". The antibody titers, each from the pooled sera of 8-10 mice, were analyzed by ELISA as well by Western blotting for binding activity against *P. aeruginosa*, and by ELISA against peptides D1-D5.

20 Fig. 2 shows that specific antibody titers against *P. aeruginosa* were obtained in all immunized groups up to serum dilutions 1:15625. Western blot analysis of the sera with *P. aeruginosa* polypeptides showed specific staining of OprI as well as of OprF by sera from all immunized groups. No staining of OprI or OprF was observed in the GST immunized control group. Further analysis of the sera against peptides D1-D5 (Fig. 3) showed that, in GST-OprF-OprI as well as 25 GST-OprI-OprF immunized animals, peptides D5 and D4 predominated. In order to test whether the induced antibodies against outer membrane fusion proteins protect mice against *P. aeruginosa* infection, mice received three doses of cyclophosphamide for immunosuppression. Leukocyte counts determined in peripheral blood samples of 15 non-immunized control animals dropped to mean levels below 400/µl. One day later, the animals were challenged with either 5 x 10<sup>1</sup>, 5 x 10<sup>2</sup>, 5 x 10<sup>3</sup> or 5 x 10<sup>4</sup> CFU of *P. aeruginosa* serogroup 1. Survival of the animals was registered for one week. 30 Fig 4 and Table 2 show the survival rates of the animals after 4 different challenge doses and the LD<sub>50</sub> values for each of the vaccines, calculated by probit regression analysis. For groups immunized with GST only or with GST-OprI-OprF, LD<sub>50</sub> values as low as 1.58 and 2.65 were calculated. Simultaneous vaccination with a mixture of GST-OprI and GST-OprF induced an increase of the LD<sub>50</sub> value to 83.3 CFU. This difference, however, was found to be not statistically significant. In contrast, after vaccination with the hybrid GST-OprF-OprI a highly significant shift of the LD<sub>50</sub> value towards 35 1540 CFU was calculated ( $p \leq 0.001$ ). Compared to the GST immunized controls, a protection value of 962 was calculated for the GST-OprF-OprI group. These results could be confirmed ( $p \leq 0.001$ ) in an identically designed second experiment.

40 Analysis of the data by the proportional hazard model and calculation of the reduction of the rise ratios induced by the different vaccine preparations is shown in Table 2. Vaccination with GST-OprF-OprI reduced the risk ratio highly significantly ( $p \leq 0.0001$ ) to a value of 0.3 compared to the GST immunized controls. Even for a challenge dose of 5 x 10<sup>3</sup> CFU, a significant ( $p \leq 0.0019$ ) reduction of the risk ratio to a value of 0.69 was calculated by backward elimination 45 for the GST-OprF-OprI vaccinated group, with reference based on GST, GST-OprF+GST-OprI, GST-OprI-OprF immunized groups, and doses one and two (5 x 10<sup>1</sup> and 5 x 10<sup>2</sup>).

**Example 5: Expression of OprF-OprI in yeast.**

45 For the expression of the OprF-OprI hybrid protein without an additional fusion component we chose as an alternative host cell *Saccharomyces cerevisiae* and as plasmid pYepsec1. OprF-OprI contained in pYepsec1-F-1 (Fig. 1) was expressed only in minute amounts in *S. cerevisiae*. Since OprF as, well as OprI are exported in *Pseudomonadaceae* through the periplasmic space, we tried to copy the export in *S. cerevisiae*. To this end, the OprF-OprI hybrid protein 50 was fused to the secretion signal sequence of the killer toxin (kt) of the yeast *Kluyveromyces lactis*. The tripartite hybrid protein kt-OprF-OprI encoded by pYepsec1-F-1 (Fig. 1) now consists of the following polypeptide stretches: first there are the 16 amino acids of the yeast secretion signal sequences, followed by 9 amino acids encoded by a DNA linker, and then followed by the OprF specific polypeptide stretch from amino acids 190-342 and an OprI peptide including 55 amino acids 21-83. The OprF specific polypeptide carries the potential glycosylation site asparagine-x-threonine (see Fig. 1) twice. These glycosylation sites should be recognizable if the fusion protein enters the secretory pathway. Upon fusion to the killer toxin leader sequence, OprF-OprI was detected in yeast cell extracts by Western blot analysis, when expressed under induced condition of the UAS<sub>GAL</sub>/CYC1 promoter; but no secreted antigen was detected in the culture broth.

The OprF-OprI fusions protein expressed in yeast did not migrate as a sharp band in SDS polyacrylamide gels, but showed a heterogeneous distribution, appearing in several smearing bands. This indicates posttranslational modification by N-glycosylation. Incubation of the recombinant *P. aeruginosa* antigen with endoglycosidase F resulted in the appearance of a sharp band of lower molecular weight, indicating the entering of OprF-OprI into the secretory pathway, when fused to the killer toxin leader sequence, and the glycosylation of at least one of the two potential glycosylation sites.

**Example 6:** Passive immunization with antibodies against yeast-derived OprF-OprI.

The recombinant *Pseudomonas* antigen was enriched from the supernatants of yeast cell extracts by ammonium salt precipitation and immunoaffinity chromatography, using an anti OprF mouse monoclonal antibody, directed against epitope D1. Rabbits were then immunized three times with the antigen, and sera were collected from the animals. Whereas the preimmune sera did not show any reactivity with either *P. aeruginosa* OprF or OprI, the sera from the immunized rabbits reacted specifically with the outer membrane proteins OprF and OprI from the three different ATCC strains of *P. aeruginosa*, as well with the three different clinical isolates of *P. aeruginosa* tested. The protective efficacy of these sera was tested in SCID mice for defence against a lethal challenge with *P. aeruginosa*. As shown in Table 3, mice injected with the control anti-yeast serum were not protected against infection even at a challenge dose of  $5 \times 10^1$  (Table 3, group 1). On the other hand, mice which received the OprF-OprI specific rabbit serum were fully protected against a  $5 \times 10^2$  CFU challenge dose of *P. aeruginosa* (Table 3, group 3), and 40 % survival was observed after challenge with  $5 \times 10^3$  CFU. As an additional control, protection by rabbit serum induced against LPS of the challenge strain, *P. aeruginosa* serogroup 1, was tested. Up to a challenge dose of  $5 \times 10^3$ , 100 % of the animals protected with LPS specific serum survived (Table 3, group 5). No survival could be observed in this group after a 10-fold higher challenge dose of  $5 \times 10^4$ . Statistical analysis was used to compare the protective doses of OprF-OprI specific serum, of LPS specific serum, and the anti-yeast control group for protection against *P. aeruginosa* infection. The results showed an 85-fold increase in potency of the OprF-OprI serum in comparison with the anti yeast serum ( $p \leq 0.002$  - see Table 3, group 3). As against this, a 325 higher potency was calculated for the LPS specific serum than for the anti-yeast serum ( $p \leq 0.001$ ).

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Table 1: Characterization of B cell epitopes of *P. aeruginosa* OprF

peptide	OprF specificaa region	MAbs* specificaa region	rabbit anti- OprF ELISA (against peptide)	rabbit anti- OprF ELISA (against OprF)	rabbit anti- "intact P. aeruginosa"**
D1	190-213	+	+	-	-
D2	212-240	+	+	-	+
D3	239-250	-	+	+	-
D4	284-216	+	+	+	-
D5	332-350	+	+	+	+

\* MAbs were induced in mice against a recombinant protein representing amino acids 58-350 of OprF, binding to peptides D1-D5 was analyzed by ELISA.

\*\* Rabbits were immunized with peptides linked to KLH.

\*\*\* estimated with *P. aeruginosa* serogroup 11 (ATCC 33359).

Table 2

Statistical analysis of survival of mice*				
	Vaccine			
	GST	GST-OprF+GST-OprI	GST-OprF-OprI	GST-OprI-OprF
LD <sub>50</sub>	1.58	83.34	1540**	2.65
Shift LD <sub>50</sub> **	1	52	962	1.7
Risk Ratio***	1	0.732	0.344***	0.889

\* mice were vaccinated with the indicated GST linked recombinant Oprs or GST as control.

\*\* LD<sub>50</sub> values were calculated by probit analysis (Finney, D. J. (1971), Probit analysis, Cambridge University Press, Cambridge).

\*\*\* P<0.05 versus GST group. \*\*\*\*P<0.0001 versus GST group.

\*\*\*\* Risk ratios were calculated by the proportional hazard model (Lawless, J. F (1982), Statistical Methods for Lifetime Data, John Wiley & Sons, New York) with reference based on GST group.

Table 3

Protection against <i>P. aeruginosa</i> infection in SCID mice by rabbit anti OprF-OprI sera							
Surviving animals after transfer of specific rabbit serum before challenge, group no. (n=5)							
challenge d se** (CFU)	1 yeast* control	2 yeast* control 1:10	3 OprF-OprI*	4 OprF-OprI* 1:10	5 <i>P. aeruginosa</i> ***	6 challenge control	7 mucin control
5 x 10 <sup>0</sup>	5	5	5	5	5	1	
5 x 10 <sup>1</sup>	1	1	5	4	5	0	
5 x 10 <sup>2</sup>	1	0	5	2	5	0	
5 x 10 <sup>3</sup>	0	1	2	0	5	0	
5 x 10 <sup>4</sup>	0	0	0	0	0	0	
mucin							5

\* Rabbit serum of animals immunized with the indicated antigen.

\*\* Female C.B-17 scid/scid mice (SCID) were challenged intraperitoneally with the indicated colony forming units (CFU) of *P. aeruginosa* serogroup 1 suspended with 0.5 ml of mucin.

\*\*\* rabbit serum of animals immunized with *P. aeruginosa* serogroup 1. Statistical analysis (probit analysis for parallel line model); group 1 versus group 3: 85-fold increase in potency, significance (chi-square), 0.002. Group 1 versus group 5; 325-fold increase in potency, significance 0.001

## 50 Legends to Figures

### Fig. 1

Schematic overview of the constructed recombinant fusion proteins of outer membrane proteins of *P. aeruginosa*.

For expression in *E. coli* K12, the vector pGEX-2a, which codes for glutathione-S-transferase was used.

█ signal sequence of *Kluyveromyces lactis* killer toxin.      potential glycosylation site.

█ GST (aa 1-225).    █ OprF (aa 190-350).

█ OprF (aa190-342)    █ OprI (aa21-83)Fig. 2

**EP 0 717 106 A1**

Determination of antibody titers against *P. aeruginosa* in sera of mice immunized with the indicated GST linked recombinant outer membrane vaccine or with GST alone. ELISA measurements were carried out on plates coated with sonicated *P. aeruginosa* serogroup 12.

5 **Fig. 3**

Antibody determination by ELISA against synthetic peptides D1-D5 listed in Table 1, which represent B-cell epitopes of OprF. Mice were immunized four times with the indicated recombinant fusion proteins or GST alone.

10 **Fig. 4**

Survival of BALB/c mice after immunization with the indicated vaccine or GST alone, followed by immunosuppression and intraperitoneal challenge with 5, 50, 500 or 5000 colony forming units of *P. aeruginosa* serogroup 1. Bars represent percentage of survivors (n=16-17) per challenge dose.

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## SEQUENCE LISTING

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## (1) GENERAL INFORMATION:

## (i) APPLICANT:

- (A) NAME: Behringwerke Aktiengesellschaft
- (B) STREET: Emil-von-Behring-Str. 76
- (C) CITY: Marburg
- (E) COUNTRY: Germany
- (F) POSTAL CODE (ZIP): 3500 40
- (G) TELEPHONE: 0 64 21-39-22 05
- (H) TELEFAX: 0 64 21-39-45 58

10

(ii) TITLE OF INVENTION: Immunogenic hybrid protein OprI-OprF derived  
15 from *P. aeruginosa* membrane proteins

15

(iii) NUMBER OF SEQUENCES: 8

20

## (iv) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Floppy disk
- (B) COMPUTER: IBM PC compatible
- (C) OPERATING SYSTEM: PC-DOS/MS-DOS
- (D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

25

## (v) CURRENT APPLICATION DATA:

APPLICATION NUMBER: EP 94120023.0

25

## (2) INFORMATION FOR SEQ ID NO: 1:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 192 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

30

(ii) MOLECULE TYPE: cDNA

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(iii) HYPOTHETICAL: NO

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(iv) ANTI-SENSE: YES

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## (vi) ORIGINAL SOURCE:

- (A) ORGANISM: *Pseudomonas aeruginosa*
- (F) TISSUE TYPE: Serotype 6; ATCC 33354

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## (ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION:1..189
- (D) OTHER INFORMATION:/note= "Sequence is coding for oprI without signal sequence"

45

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

AGC	AGC	CAC	TCC	AAA	GAA	ACC	GAA	GCT	CGT	CTG	ACC	GCT	ACC	GAA	GAC		48
Ser	Ser	His	Ser	Lys	Glu	Thr	Glu	Ala	Arg	Leu	Thr	Ala	Thr	Glu	Asp		
1				5				10				15					

50

GCA	GCT	GCT	CGT	GCT	CAG	GCT	CGC	GAC	GAA	GCC	TAT	CGC	AAG	GCT		96
Ala	Ala	Ala	Arg	Ala	Gln	Ala	Arg	Ala	Asp	Glu	Ala	Tyr	Arg	Lys	Ala	
20				25							30					

55

GAC	GAA	GCT	CTG	GGC	GCT	GCT	CAG	AAA	GCT	CAG	CAG	ACC	GCT	GAC	GAG		144
Asp	Glu	Ala	Leu	Gly	Ala	Ala	Gln	Lys	Ala	Gln	Gln	Thr	Ala	Asp	Glu		
35				40							45						

5 GCT AAC GAG CGT GCC CTG CGC ATG CTG GAA AAA GCC AGC CGC AAG 189  
 Ala Asn Glu Arg Ala Leu Arg Met Leu Glu Lys Ala Ser Arg Lys  
 50 55 60

TAA 192

10 (2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 63 amino acids  
 (B) TYPE: amino acid  
 (D) TOPOLOGY: linear

15 (ii) MOLECULE TYPE: protein  
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Ser Ser His Ser Lys Glu Thr Glu Ala Arg Leu Thr Ala Thr Glu Asp  
 1 5 10 15

20 Ala Ala Ala Arg Ala Gln Ala Arg Ala Asp Glu Ala Tyr Arg Lys Ala  
 20 25 30

25 Asp Glu Ala Leu Gly Ala Ala Gln Lys Ala Gln Gln Thr Ala Asp Glu  
 35 40 45

Ala Asn Glu Arg Ala Leu Arg Met Leu Glu Lys Ala Ser Arg Lys  
 50 55 60

25 (2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 486 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: double  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

35 (iv) ANTI-SENSE: YES

(vi) ORIGINAL SOURCE:

(A) ORGANISM: *Pseudomonas aeruginosa*  
 (F) TISSUE TYPE: Serotype 6; ATCC 33354

40 (ix) FEATURE:

(A) NAME/KEY: CDS  
 (B) LOCATION: 1..483  
 (D) OTHER INFORMATION:/note= "Sequence is coding for oprF  
 C-terminus"

45 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

GCT CCG GCT CCG GAA CCG GTT GCC GAC GTT TGC TCC GAC TCC GAC AAC 48  
 Ala Pro Ala Pro Glu Pro Val Ala Asp Val Cys Ser Asp Ser Asp Asn  
 65 70 75

50 GAC GGC GTC TGC GAC AAC GTC GAC AAG TGC CCG GAC ACC CCG GCC AAC 96  
 Asp Gly Val Cys Asp Asn Val Asp Lys Cys Pro Asp Thr Pro Ala Asn  
 80 85 90 95

GTC ACC GTT GAC GCC AAC GGC TGC CCG GCT GTC GCC GAA GTC GTA CGC 144  
 Val Thr Val Asp Ala Asn Gly Cys Pro Ala Val Ala Glu Val Val Arg  
 100 105 110

5	GTA CAG CTG GAC GTG AAG TTC GAC TTC GAC AAG TCC AAG GTC AAA GAG Val Gln Leu Asp Val Lys Phe Asp Phe Asp Lys Ser Lys Val Lys Glu 115 120 125	192
10	AAC AGC TAC GCT GAC ATC AAG AAC CTG GCC GAC TTC ATG AAG CAG TAC Asn Ser Tyr Ala Asp Ile Lys Asn Leu Ala Asp Phe Met Lys Gln Tyr 130 135 140	240
15	CCG TCC ACT TCC ACC ACC GTT GAA GGT CAT ACC GAC TCC GTC GGT ACC Pro Ser Thr Ser Thr Thr Val Glu Gly His Thr Asp Ser Val Gly Thr 145 150 155	288
20	GAC GCT TAC AAC CAG AAG CTG TCC GAG CGT CGT GCC AAC GCC GTT CGT Asp Ala Tyr Asn Gln Lys Leu Ser Glu Arg Arg Ala Asn Ala Val Arg 160 165 170 175	336
25	GAC GTA CTG GTC AAC GAG TAC GGT GTG GAA GGT GGT CGC GTG AAC GCT Asp Val Leu Val Asn Glu Tyr Gly Val Glu Gly Gly Arg Val Asn Ala 180 185 190	384
30	GTC GGT TAC GGC GAG TCC CGC CCG GTT GCC GAC AAC GCC ACC GCT GAA Val Gly Tyr Gly Glu Ser Arg Pro Val Ala Asp Asn Ala Thr Ala Glu 195 200 205	432
35	GGC CGC GCT ATC AAC CGT CGC GTT GAA GCC GAA GTA GAA GCC GAA GCC Gly Arg Ala Ile Asn Arg Arg Val Glu Ala Glu Val Glu Ala Glu Ala 210 215 220	480
40	AAG TAA Lys	486

## (2) INFORMATION FOR SEQ ID NO: 4:

30	(i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 161 amino acids (B) TYPE: amino acid (C) TOPOLOGY: linear
35	(ii) MOLECULE TYPE: protein (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:
40	Ala Pro Ala Pro Glu Pro Val Ala Asp Val Cys Ser Asp Ser Asp Asn 1 5 10 15
45	Asp Gly Val Cys Asp Asn Val Asp Lys Cys Pro Asp Thr Pro Ala Asn 20 25 30
50	Val Thr Val Asp Ala Asn Gly Cys Pro Ala Val Ala Glu Val Val Arg 35 40 45
55	Val Gln Leu Asp Val Lys Phe Asp Phe Asp Lys Ser Lys Val Lys Glu 50 55 60
60	Asn Ser Tyr Ala Asp Ile Lys Asn Leu Ala Asp Phe Met Lys Gln Tyr 65 70 75 80
65	Pro Ser Thr Ser Thr Val Glu Gly His Thr Asp Ser Val Gly Thr 85 90 95
70	Asp Ala Tyr Asn Gln Lys Leu Ser Glu Arg Arg Ala Asn Ala Val Arg 100 105 110
75	Asp Val Leu Val Asn Glu Tyr Gly Val Glu Gly Gly Arg Val Asn Ala 115 120 125

5 Val Gly Tyr Gly Glu Ser Arg Pro Val Ala Asp Asn Ala Thr Ala Glu  
 130 135 140

Gly Arg Ala Ile Asn Arg Arg Val Glu Ala Glu Val Glu Ala Glu Ala  
 145 150 155 160

Lys

10 (2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 645 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: double  
 (D) TOPOLOGY: linear

15 (ii) MOLECULE TYPE: cDNA

20 (iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

25 (vi) ORIGINAL SOURCE:

(A) ORGANISM: *Pseudomonas aeruginosa*  
 (F) TISSUE TYPE: Serotype 6; ATCC 33354

(ix) FEATURE:

(A) NAME/KEY: CDS  
 (B) LOCATION: 1..642  
 (D) OTHER INFORMATION:/note= "Sequence is coding for oprF  
 C-terminus and oprI without signal sequence"

30 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

GCT CCG GAA CCG GTT GCC GAC GTT TGC TCC GAC TCC GAC AAC GAC GGC  
 Ala Pro Glu Pro Val Ala Asp Val Cys Ser Asp Ser Asp Asn Asp Gly  
 165 170 175

48

GTC TGC GAC AAC GTC GAC AAG TGC CCG GAC ACC CCG GCC AAC GTC ACC  
 Val Cys Asp Asn Val Asp Lys Cys Pro Asp Thr Pro Ala Asn Val Thr  
 180 185 190

96

GTT GAC GCC AAC GGC TGC CCG GCT GTC GCC GAA GTC GTA CGC GTA CAG  
 Val Asp Ala Asn Gly Cys Pro Ala Val Ala Glu Val Val Arg Val Gln  
 195 200 205

144

40 CTG GAC GTG AAG TTC GAC TTC GAC AAG TCC AAG GTC AAA GAG AAC AGC  
 Leu Asp Val Lys Phe Asp Phe Asp Lys Ser Lys Val Lys Glu Asn Ser  
 210 215 220 225

192

TAC GCT GAC ATC AAG AAC CTG GCC GAC TTC ATG AAG CAG TAC CCG TCC  
 Tyr Ala Asp Ile Lys Asn Leu Ala Asp Phe Met Lys Gln Tyr Pro Ser  
 230 235 240

240

45 ACT TCC ACC ACC GTT GAA GGT CAT ACC GAC TCC GTC GGT ACC GAC GCT  
 Thr Ser Thr Thr Val Glu Gly His Thr Asp Ser Val Gly Thr Asp Ala  
 245 250 255

288

50 TAC AAC CAG AAG CTG TCC GAG CGT CGT GCC AAC GCC GTT CGT GAC GTA  
 Tyr Asn Gln Lys Leu Ser Glu Arg Arg Ala Asn Ala Val Arg Asp Val  
 260 265 270

336

CTG GTC AAC GAG TAC GGT GTG GAA GGT CGC GTG AAC GCT GTC GGT  
 Leu Val Asn Glu Tyr Gly Val Glu Gly Arg Val Asn Ala Val Gly

384

	275	280	285	
5	TAC GGC GAG TCC CGC CCG GTT GCC GAC AAC GCC ACC GCT GAA GGC CGC			432
	Tyr Gly Glu Ser Arg Pro Val Ala Asp Asn Ala Thr Ala Glu Gly Arg			
	290 295 300 305			
10	GCT ATC AAC CGT CGC GTT GAA AGC AGC CAC TCC AAA GAA ACC GAA GCT			480
	Ala Ile Asn Arg Arg Val Glu Ser Ser His Ser Lys Glu Thr Glu Ala			
	310 315 320			
15	CGT CTG ACC GCT ACC GAA GAC GCA GCT GCT CGT GCT CAG GCT CGC GCT			528
	Arg Leu Thr Ala Thr Glu Asp Ala Ala Ala Arg Ala Gln Ala Arg Ala			
	325 330 335			
20	GAC GAA GCC TAT CGC AAG GCT GAC GAA GCT CTG GGC GCT GCT CAG AAA			576
	Asp Glu Ala Tyr Arg Lys Ala Asp Glu Ala Leu Gly Ala Ala Gln Lys			
	340 345 350			
25	GCT CAG CAG ACC GCT GAC GAG GCT AAC GAG CGT GCC CTG CGC ATG CTG			624
	Ala Gln Gln Thr Ala Asp Glu Ala Asn Glu Arg Ala Leu Arg Met Leu			
	355 360 365			
30	GAA AAA GCC AGC CGC AAG TAA			645
	Glu Lys Ala Ser Arg Lys			
	370 375			

## (2) INFORMATION FOR SEQ ID NO: 6:

25	(i) SEQUENCE CHARACTERISTICS:			
	(A) LENGTH: 214 amino acids			
	(B) TYPE: amino acid			
	(D) TOPOLOGY: linear			
30	(ii) MOLECULE TYPE: protein			
	(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:			
	Ala Pro Glu Pro Val Ala Asp Val Cys Ser Asp Ser Asp Asn Asp Gly			
	1 5 10 15			
35	Val Cys Asp Asn Val Asp Lys Cys Pro Asp Thr Pro Ala Asn Val Thr			
	20 25 30			
	Val Asp Ala Asn Gly Cys Pro Ala Val Ala Glu Val Val Arg Val Gln			
	35 40 45			
40	Leu Asp Val Lys Phe Asp Phe Asp Lys Ser Lys Val Lys Glu Asn Ser			
	50 55 60			
	Tyr Ala Asp Ile Lys Asn Leu Ala Asp Phe Met Lys Gln Tyr Pro Ser			
	65 70 75 80			
	Thr Ser Thr Thr Val Glu Gly His Thr Asp Ser Val Gly Thr Asp Ala			
	85 90 95			
45	Tyr Asn Gln Lys Leu Ser Glu Arg Arg Ala Asn Ala Val Arg Asp Val			
	100 105 110			
	Leu Val Asn Glu Tyr Gly Val Glu Gly Gly Arg Val Asn Ala Val Gly			
	115 120 125			
50	Tyr Gly Glu Ser Arg Pro Val Ala Asp Asn Ala Thr Ala Glu Gly Arg			
	130 135 140			
	Ala Ile Asn Arg Arg Val Glu Ser Ser His Ser Lys Glu Thr Glu Ala			
	145 150 155 160			

5 Arg Leu Thr Ala Thr Glu Asp Ala Ala Ala Arg Ala Gln Ala Arg Ala  
 165 170 175  
 Asp Glu Ala Tyr Arg Lys Ala Asp Glu Ala Leu Gly Ala Ala Gln Lys  
 180 185 190  
 Ala Gln Gln Thr Ala Asp Glu Ala Asn Glu Arg Ala Leu Arg Met Leu  
 195 200 205  
 10 Glu Lys Ala Ser Arg Lys  
 210

## (2) INFORMATION FOR SEQ ID NO: 7:

## 15 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 681 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

20 (ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

25 (vi) ORIGINAL SOURCE:

- (A) ORGANISM: *Pseudomonas aeruginosa*
- (F) TISSUE TYPE: Serotype 6; ATCC 33354

30 (ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 1..678
- (D) OTHER INFORMATION:/note= "Sequence is coding for oprI without signal sequence and oprF C-terminus"

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

35	AGC AGC CAC TCC AAA GAA ACC GAA GCT CGT CTG ACC GCT ACC GAA GAC Ser Ser His Ser Lys Glu Thr Glu Ala Arg Leu Thr Ala Thr Glu Asp 215 220 225 230	48
40	CA GCT GCT CGT GCT CAG GCT CGC GCT GAC GAA GCC TAT CGC AAG GCT Ala Ala Ala Arg Ala Gln Ala Arg Ala Asp Glu Ala Tyr Arg Lys Ala 235 240 245	96
45	GAC GAA GCT CTG GGC GCT GCT CAG AAA GCT CAG CAG ACC GCT GAC GAG Asp Glu Ala Leu Gly Ala Ala Gln Lys Ala Gln Gln Thr Ala Asp Glu 250 255 260	144
50	GCT AAC GAG CGT GCC CTG CGC ATG CTG GAA AAA GCC AGC CGC AAG GAG Ala Asn Glu Arg Ala Leu Arg Met Leu Glu Lys Ala Ser Arg Lys Glu 265 270 275	192
55	CTC GCT CCG GCT CCG GAA CCG GTT GCC GAC GTT TGC TCC GAC TCC GAC Leu Ala Pro Ala Pro Glu Pro Val Ala Asp Val Cys Ser Asp Ser Asp 280 285 290	240
	AAC GAC GGC GTC TGC GAC AAC GTC GAC AAG TGC CCG GAC ACC CCG GCC Asn Asp Gly Val Cys Asp Asn Val Asp Lys Cys Pro Asp Thr Pro Ala 295 300 305 310	288
	AAC GTC ACC GTT GAC GCC AAC GGC TGC CCG GCT GTC GCC GAA GTC GTA Asn Val Thr Val Asp Ala Asn Gly Cys Pro Ala Val Ala Glu Val Val 315 320 325	336

5	CGC GTA CAG CTG GAC GTG AAG TTC GAC TTC GAC AAG TCC AAG GTC AAA Arg Val Gln Leu Asp Val Lys Phe Asp Phe Asp Lys Ser Lys Val Lys 330 335 340	384
10	GAG AAC AGC TAC GCT GAC ATC AAG AAC CTG GCC GAC TTC ATG AAG CAG Glu Asn Ser Tyr Ala Asp Ile Lys Asn Leu Ala Asp Phe Met Lys Gln 345 350 355	432
15	TAC CCG TCC ACT TCC ACC ACC GTT GAA GGT CAT ACC GAC TCC GTC GGT Tyr Pro Ser Thr Ser Thr Val Glu Gly His Thr Asp Ser Val Gly 360 365 370	480
20	ACC GAC GCT TAC AAC CAG AAG CTG TCC GAG CGT CGT GCC AAC GCC GTT Thr Asp Ala Tyr Asn Gln Lys Leu Ser Glu Arg Arg Ala Asn Ala Val 375 380 385 390	528
25	CGT GAC GTA CTG GTC AAC GAG TAC GGT GTG GAA GGT GGT CGC GTG AAC Arg Asp Val Leu Val Asn Glu Tyr Gly Val Glu Gly Arg Val Asn 395 400 405	576
30	GCT GTC GGT TAC GGC GAG TCC CGC CCG GTT GCC GAC AAC GCC ACC GCT Ala Val Gly Tyr Gly Glu Ser Arg Pro Val Ala Asp Asn Ala Thr Ala 410 415 420	624
35	GAA GGC CGC GCT ATC AAC CGT CGC GTT GAA GCC GAA GTA GAA GCC GAA Glu Gly Arg Ala Ile Asn Arg Arg Val Glu Ala Glu Val Glu Ala Glu 425 430 435	672
40	GCC AAG TAA Ala Lys 440	681

## (2) INFORMATION FOR SEQ ID NO: 8:

30	(i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 226 amino acids (B) TYPE: amino acid (D) TOPOLOGY: linear
35	(ii) MOLECULE TYPE: protein (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:
40	Ser Ser His Ser Lys Glu Thr Glu Ala Arg Leu Thr Ala Thr Glu Asp 1 5 10 15
45	Ala Ala Ala Arg Ala Gln Ala Arg Ala Asp Glu Ala Tyr Arg Lys Ala 20 25 30
50	Asp Glu Ala Leu Gly Ala Ala Gln Lys Ala Gln Gln Thr Ala Asp Glu 35 40 45
55	Ala Asn Glu Arg Ala Leu Arg Met Leu Glu Lys Ala Ser Arg Lys Glu 50 55 60
60	Leu Ala Pro Ala Pro Glu Pro Val Ala Asp Val Cys Ser Asp Ser Asp 65 70 75 80
65	Asn Asp Gly Val Cys Asp Asn Val Asp Lys Cys Pro Asp Thr Pro Ala 85 90 95
70	Asn Val Thr Val Asp Ala Asn Gly Cys Pro Ala Val Ala Glu Val Val 100 105 110
75	Arg Val Gln Leu Asp Val Lys Phe Asp Phe Asp Lys Ser Lys Val Lys 115 120 125

25

30 1. A hybrid protein comprising the *Pseudomonas aeruginosa* outer membrane protein I which is fused with its amino terminal end to the carboxy terminal end of a carboxy terminal portion of the *Pseudomonas aeruginosa* outer membrane protein F, said carboxy terminal portion comprising the sequence from aa 190 to aa 350.

2. The hybrid protein as claimed in claim 1, wherein said carboxy terminal portion is the sequence from aa 190 to aa 342.

35 3. A hybrid protein comprising the *Pseudomonas aeruginosa* outer membrane protein I which is fused with its amino terminal end to the carboxy terminal end of a carboxy terminal portion of the *Pseudomonas aeruginosa* outer membrane protein F, said carboxy terminal portion comprising at least one surface-exposed B-cell epitope selected from the group consisting of SEE 1, SEE 2, SEE 3 and SEE 4.

40 4. A vaccine comprising a hybrid protein as claimed in claim 1, 2 or 3.

5. Monoclonal or polyclonal antibodies against the hybrid protein as claimed in claim 1, 2 or 3.

45 6. A vaccine comprising the antibodies as claimed in claim 5.

7. A nucleic acid, coding for the hybrid protein as claimed in claim 1, 2 or 3.

8. A process for the preparation of the hybrid protein as claimed in claim 1, 2 or 3, which comprises bringing about the expression of the nucleic acid as claimed in claim 7 in pro- or eukaryotic cells.

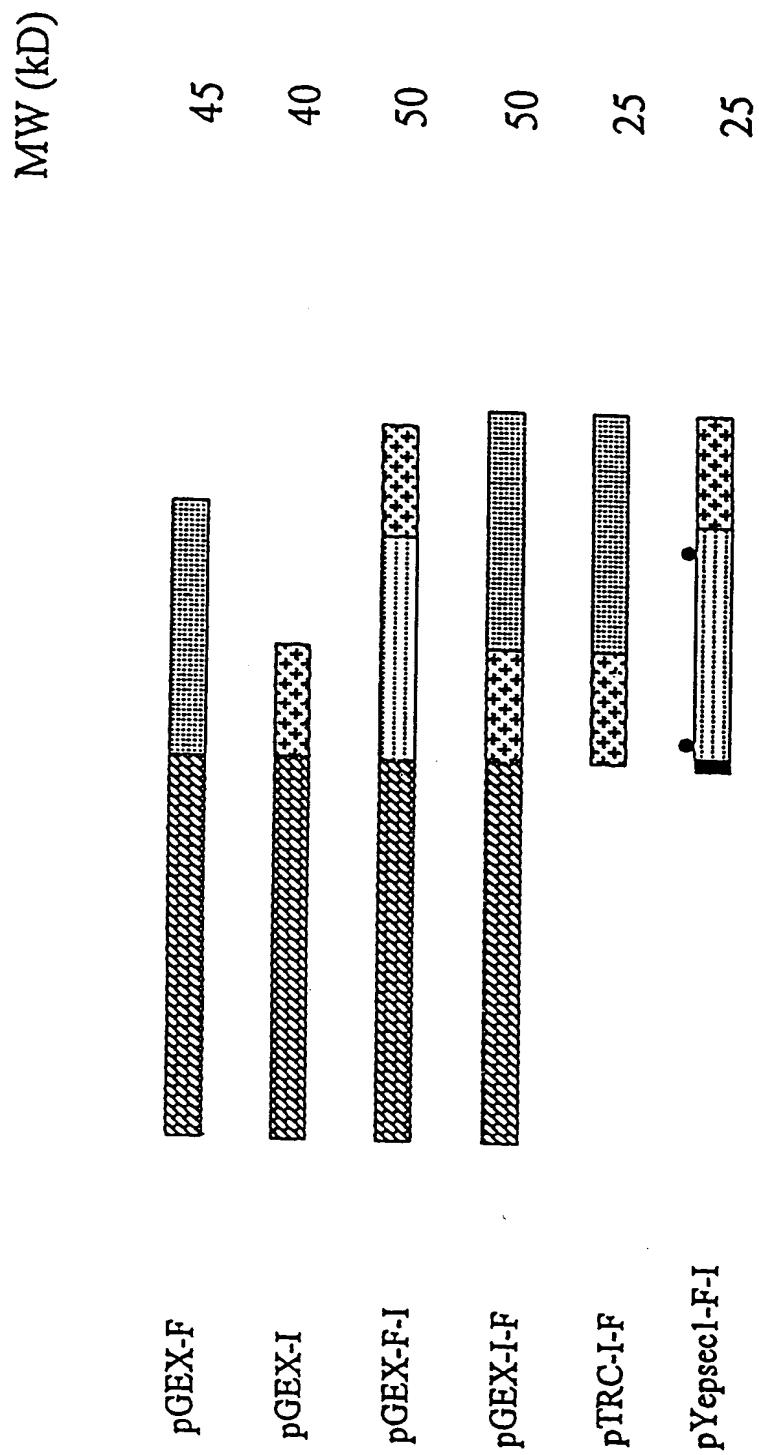


FIG. 1

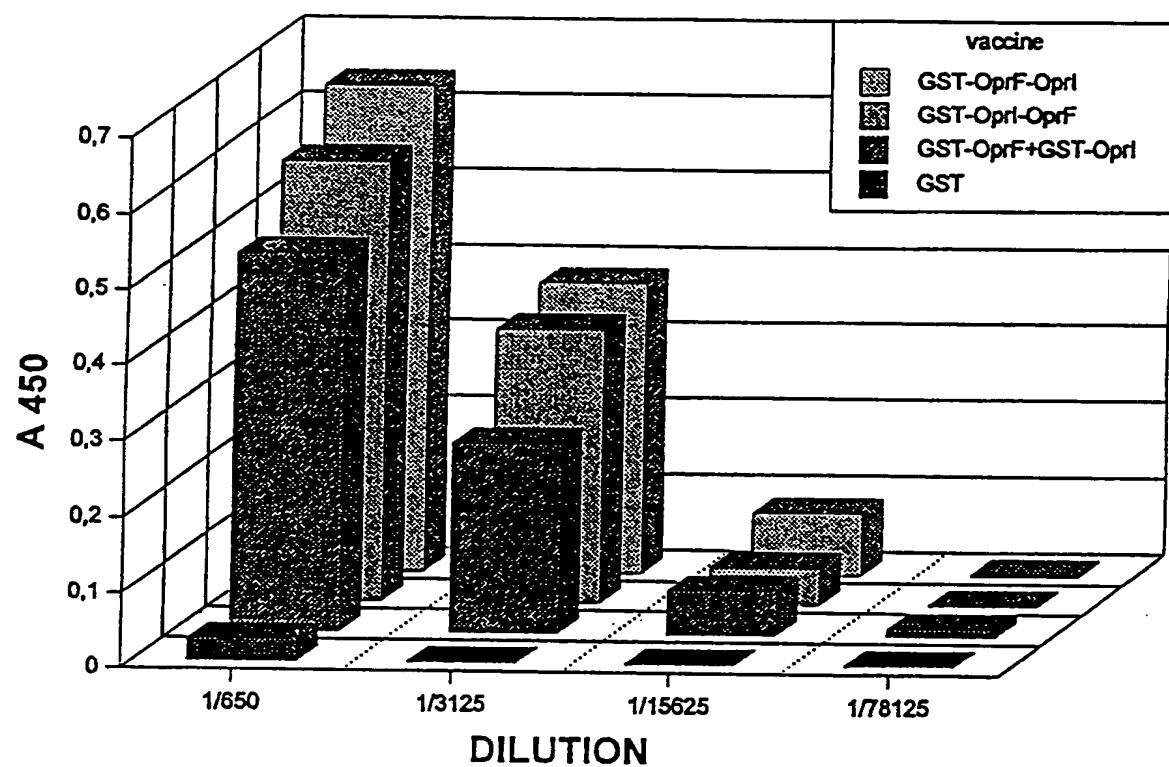
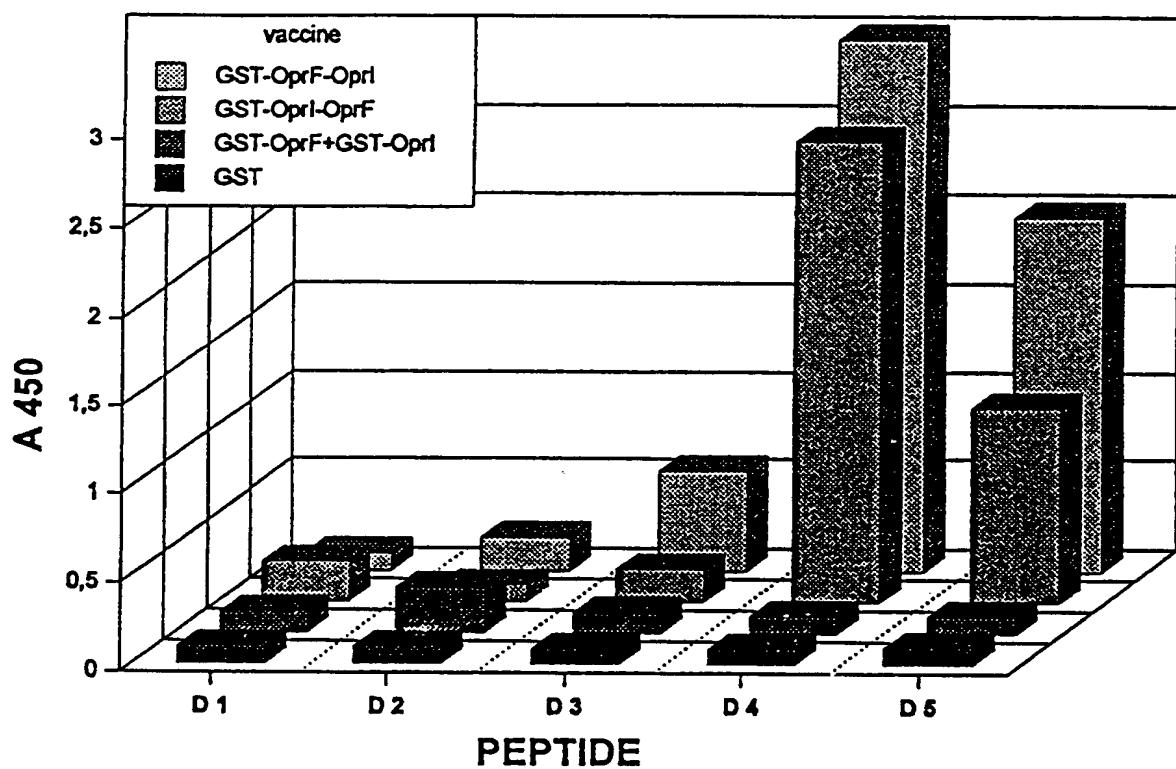
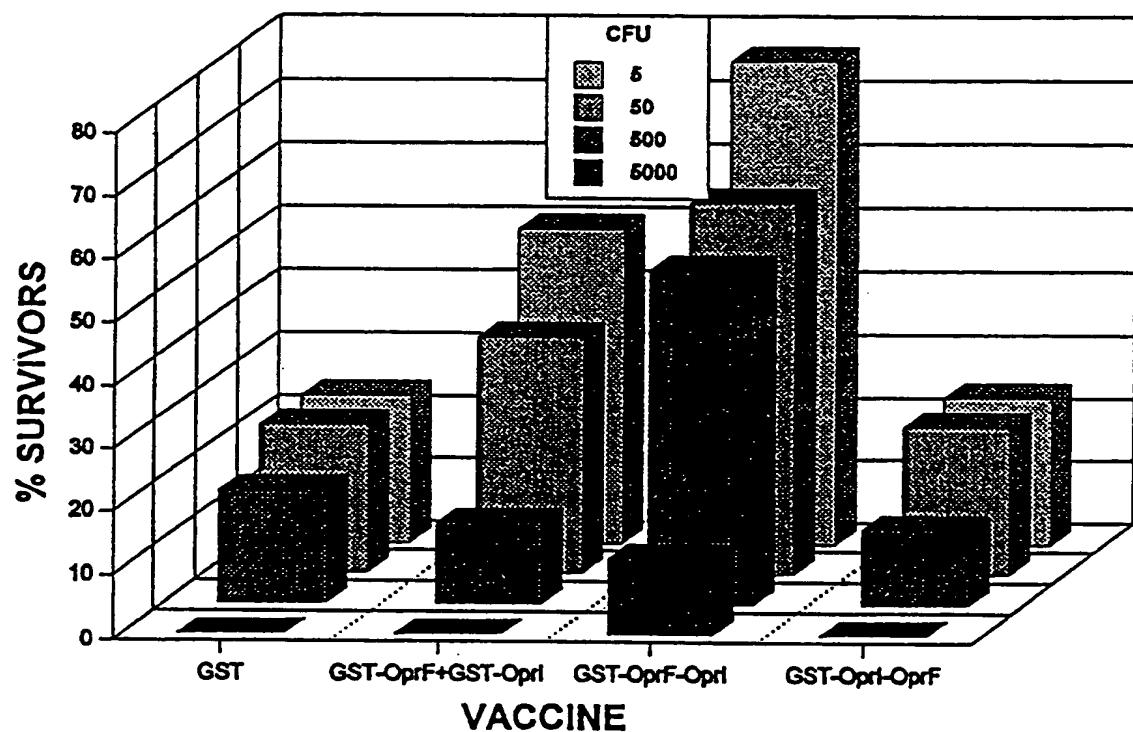


FIG. 2



**FIG.3**



**FIG.4**



## EUROPEAN SEARCH REPORT

Application Number  
EP 95 11 8098

DOCUMENTS CONSIDERED TO BE RELEVANT					
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.)		
X	BEHRING INST. MITT., vol. 95, pages 85-96, VON SPECHT ET AL. 'Outer membrane proteins of <i>Pseudomonas aeruginosa</i> as vaccine candidates' * the whole document * ---	1-8	C12N15/31 C12N15/62 C07K14/21 C07K19/00 C07K16/12 A61K39/104 //(C07K14/21, C12R1:385)		
Y	J. BACTERIOL., vol. 174, no. 15, pages 4977-4985, FINNEN ET AL. 'Analysis of the <i>Pseudomonas aeruginosa</i> major outer membrane protein OprF by use of truncated OprF derivatives and monoclonal antibodies' * abstract * * page 4982 * * page 4983, right column * * page 4984 * ---	1,2,4-8			
Y,D	INFECT. IMMUN., vol. 59, no. 4, pages 1251-1254, FINKE ET AL. 'Protection of immunosuppressed mice against infection with <i>Pseudomonas aeruginosa</i> by recombinant P-aeruginosa Lipoprotein I and lipoprotein I-specific monoclonal antibodies' * the whole document * ---	1,2,4-8 -/-	TECHNICAL FIELDS SEARCHED (Int.Cl.) C12N C07K		
The present search report has been drawn up for all claims					
Place of search	Date of completion of the search	Examiner			
THE HAGUE	5 March 1996	Gac, G			
CATEGORY OF CITED DOCUMENTS					
X : particularly relevant if taken alone	T : theory or principle underlying the invention				
Y : particularly relevant if combined with another document of the same category	E : earlier patent document, but published on, or after the filing date				
A : technological background	D : document cited in the application				
O : non-written disclosure	L : document cited for other reasons				
P : intermediate document	& : member of the same patent family, corresponding document				



European Patent  
Office

## EUROPEAN SEARCH REPORT

Application Number  
EP 95 11 8098

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.6)
A,D	<p>INFECT. IMMUN., vol. 60, no. 9, pages 3497-3503, HUGHES ET AL. 'Synthetic peptides representing epitopes of outer membrane protein F of <i>Pseudomonas aeruginosa</i> that elicit antibodies reactive with whole cells of heterologous immunotype strains of <i>P. aeruginosa</i>' * the whole document *</p> <p>---</p>	1-8	
	<p>WO-A-93 24636 (THE UNIVERSITY OF BRITISH COLUMBIA) 9 December 1993 * the whole document *</p> <p>-----</p>	1-8	
			TECHNICAL FIELDS SEARCHED (Int.Cl.6)
<p>The present search report has been drawn up for all claims</p>			
Place of search	Date of completion of the search	Examiner	
THE HAGUE	5 March 1996	Gac, G	
CATEGORY OF CITED DOCUMENTS		<p>T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons ..... &amp; : member of the same patent family, corresponding document</p>	
<p>X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document</p>			

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